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This proposal describes a body of research focused on the identification and development of peptides as breast tumor imaging and therapeutic agents. Peptides that bound the breast carcinoma associated Thomsen-Friedenreich (T) antigen and erbB-2 receptor were isolated from random peptide bacteriophage display libraries. A 15 amino acid peptide (P30) and a 6 amino acid peptide (P6.1) were shown to specifically bind T antigen and the erbB-2 extracellular domain, respectively. Both peptides bound breast carcinoma cells, but not normal human endothelial cells *in vitro*. The P30 peptide was shown to inhibit breast carcinoma cell homotypic aggregation and breast carcinoma endothelial cell adhesion. Tumor cell aggregation and adhesion to endothelial cells are thought to be important processes in metastasis. Inhibitors of these processes may provide important leads in the development of anti-metastatic therapeutics. Peptide 6.1 exhibited high specificity and affinity for breast cancer cells expressing erbB-2 on their surfaces. Analogs of P6.1 will be radiolabeled and examined for their potentials as breast tumor imaging or therapeutic agents.

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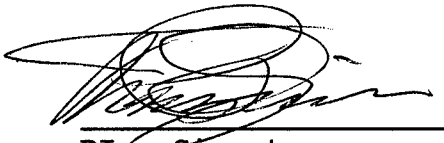

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TABLE OF CONTENTS:

INTRODUCTION:

The focus of our DoD funded research program is to identify peptides that specifically bind two breast tumor antigens, the Thomsen-Friedenreich (T) antigen and the ErbB-2 receptor, radiolabel the peptides with ^{99m}Tc and ^{188}Re, and determine the abilities of the radiolabeled peptides to target breast tumors in a scid mouse model system. The goal of this work is to develop breast tumor-avid peptides as potential *in vivo* imaging or therapeutic agents.

Background of previous work: T antigen is a disaccharide (Galß1→3GalNAc) which is masked on the surface of healthy cells, but is exposed and immuno-reactive on the surfaces of most tumor cells (1), including breast carcinoma cells (2). Peptides that bound T antigen were identified from a random peptide phage display library, screened with two glycoproteins which display T antigen on their surfaces, asialofetuin and a BSA-T conjugate. The peptide sequence with the best T antigen binding specificity and affinity was P30, His-Gly-Arg-Phe-Ile-Leu-Pro-Trp-Trp-Tyr-Ala-Phe-Ser-Pro-Ser. We had previously reported that P30 exhibited a 0.1 μM K_d for T antigen-bearing glycoproteins and bound MDA-MB435 breast carcinoma cells *in vitro* (3). The ^{99m}Tc-radiolabeled P30 peptide also bound breast tumor cells *in vitro*, but exhibited poor pharmacokinetics *in vivo*. *In vivo* clearance was slow and proceeded via hepatobiliary and GI paths. These results indicated that the radiolabeled P30 was not suitable for development into a breast cancer diagnostic agent. However, recent results indicate that the peptide itself possess anti-metastatic properties which may lead toward the development of breast carcinoma therapeutics.

The second research aim focuses on the identification of novel peptides that bind the erbB-2 receptor (4). ErbB-2, also known as HER-2 *or* neu is probably one of the best-studied proto-oncogenes frequently altered in human cancers. The erbB-2/HER-2/neu gene encodes a 185 kDa protein denoted p185, which can also be referred to as erb-2/HER-2/neu. Over expression of erbB-2 is associated with increased rates of tumor growth, enhanced rates of metastases, shorter disease-free survival, and reduced overall survival rates (4). Although erbB-2 is expressed at low levels in several normal organs and tissues (4), the elevated levels of erbB-2 in many human malignancies including breast carcinomas, and its extracellular accessibility makes it a very attractive target for the development of specific tumor agents. We previously described the identification of two peptide sequences that bound the extracellular domain of the erbB-2 receptor using random peptide bacteriophage display technology (5). The peptide sequences were chemically synthesized and examined for erbB-2 binding affinities and specificities. Peptide 6.1 (P 6.1), Lys-Cys-Cys-Tyr-Ser-Leu, exhibited optimal binding properties for the extracellular domain of erbB-2. The most recent results obtained with this peptide are reported below.

BODY:

I. T Antigen Project:

We have demonstrated that the P30 peptide bound T antigen containing glycoproteins and T antigen expressing breast carcinoma cells *in vitro*(3). Unfortunately, ^{99m}Tc-radiolabeled P30 displayed poor *in vivo* pharmacokinetics which were not consistent with further development into a diagnostic or therapeutic radiopharmaceutical. Alternatively, we hypothesized that the peptide itself might possess potential therapeutic properties based on its ability to mask T

antigen, an important cell surface ligand involved in cell adhesion. Tumor cell adhesion is an important step in metastasis. Understanding the molecular underpinnings of cancer metastasis is an important goal of modern cancer research. Metastasis is a multi-step process involving many cell-cell and cell-extracellular matrix interactions. Several of these steps include interactions between cell surface molecules such as carbohydrates, lectins, and extracellular matrix proteins participating in cell-cell recognition and adhesion (6, 7). While the initial steps of metastasis include detachment of malignant cells from the primary tumor and migration into the circulatory system, subsequent steps involve malignant cells adhering to each other (homotypic aggregation) or to host cells (heterotypic adhesion) (8-11) to form multicellular aggregates. Eventually, the circulating tumor cells become either non-specifically lodged in small capillaries or specifically bind to capillary endothelial cells and exposed basement membrane proteins, resulting in the formation of secondary tumor sites. It has been suggested that cellular adhesion is in part mediated by specific interactions between cell surface lectins and carbohydrates present on glycoproteins, glycolipids, and glycosaminoglycans (7, 9, 12,).

Involvement of Thomsen-Friedenreich (T) antigen in homotypic aggregation of MDA-MB-435 human breast carcinoma cells. Multicellular aggregate formation is an important feature of metastatic cancer cells directly correlating with their increased survival potential *in vitro* (13) and metastatic propensity *in vivo* (14). The cancer associated T antigen has been implicated in tumor cell adhesion through carbohydrate-lectin interactions (1, 15). We previously reported the expression of large quantities of T antigen on the surface of MDA-MB-435 cells confirmed by binding of T antigen specific PNA lectin (3). In this study, we investigated the role of T antigen in homotypic aggregation of the MDA-MB-435 breast cancer cells. Tumor cells collected from subconfluent (70-80%) cultures were allowed to form multicellular aggregates. The direct binding of T antigen specific PNA lectin conjugated to horseradish peroxidase followed by color reaction with DAB was used to visualize T antigen. The cytochemical analysis of the samples containing multicellular aggregates revealed significant accumulation of T antigen at the sites of cell contacts (Fig. 1 A), suggesting participation of T antigen in homotypic aggregation of MDA-MB-435 breast carcinoma cells. Consistent with this is the fact that addition of different concentrations of synthetic T antigen specific peptide, P-30 (HGRFILPWWYAFSPS), inhibited homotypic aggregation of MDA-MB-435 cells in a dose dependent manner (Fig. 1 B). A maximum inhibitory effect (over 70%) was achieved at a peptide concentration of 0.1 mg/ml. The control peptide (RRLLFYKYVYKRYRAGKQRG) which does not interact with T antigen (3) failed to inhibit homotypic aggregation of MDA-MB-435 cells (Fig. 1 C, D, and E). These findings, as well as the previously reported ability of P-30 to inhibit asialofetuin mediated aggregation of mouse melanoma cells (3) suggest that the effect of P-30 on homotypic aggregation of MDA-MB-435 cells is T antigen specific.

Adhesion of MDA-MB-435 breast carcinoma cells to the endothelium. Since T antigen (Galß1→3GalNAc) has β-galactose as a terminal sugar, it is likely that T antigen mediated interactions may involve participation of β-galactoside specific lectins (galectins). Thus, it was of interest to analyze whether β-galactoside specific lectins participate in adhesion of the MDA-MB-435 cells to the endothelium. Confocal laser microscopy revealed clustering of both galectin-

1 and galectin-3 to the sites of contact between MDA-MB-435 cells and human umbilical endothelial cells (Fig. 2 A and B) indicative of their involvement in the interaction between cancer and endothelial cells. We could not observe, however, any signs of galectin-4 participation in this process consistent with the data on its low level of expression in MDA-MB-435 cells. Interestingly, galectin-1 and galectin-3 reacted differently on tumor and endothelial cells. A strong galectin-1 signal accumulated at the sites of tumor-endothelial cell contact predominantly on the cancer cells (Fig. 2 A) suggesting the involvement of one or more of its cognate ligands on the endothelium. Galectin-3 in contrast, while also being strongly expressed on the tumor cells, clearly demonstrated signal accumulation toward the sites of the cell contact on HUVEC (Fig. 2 B) possibly interacting with T antigen or the other putative ligands on cancer cells. We hypothesized that if galectin-3 on the endothelial cells interacts with T antigen on MDA-MB-435 cells then T antigen specific P-30 should inhibit this interaction as it did in the case of homotypic aggregation. Thus we performed experiments in which cancer cells were allowed to adhere to a monolayer of endothelial cells in the presence of P-30 (0.1 mg/ml final concentration) or a control peptide of identical concentration. The results of these experiments (Fig. 2 C, D, and E) showed that the control peptide did not effect the adhesion of the MDA-MB-435 cells to the endothelial cells (Fig. 2 C and D) while T antigen specific P-30 significantly inhibited it (Fig. 2 E). When adhesion experiments were performed with different concentrations of P-30 we found the peptide's effect to be dose dependent with the maximal inhibition achieved up to 50% (Fig. 3). These data demonstrated that adhesion of the MDA-MB-435 human breast carcinoma cells to the endothelial cells was at least in part mediated by T antigen.

II. ErbB-2 Receptor-Avid Peptide Characterization:

Peptide sequences, originally identified from random peptide bacteriophage libraries affinity selected with recombinant erbB-2 extracellular domain, where chemically synthesized using Fmoc solid phase peptide synthesis (Table 1). The peptides were cleaved from the support resin, deprotected, and purified by high performance liquid chromatography (HPLC). Mass spectrometry was used to confirm the expected molecular weights of the synthetic peptides. N-terminally biotinylated analogs of the peptide sequences were also synthesized as described above. Due to the limited solubility of P15 and biotinylated P15 in aqueous solutions, we have focus on the characterization of P6.1

Table 1. Peptide Sequences Identified from Bacteriophage Display Libraries.

Peptide Name	Peptide Sequence
P6.1	Lys-Cys-Cys-Tyr-Ser-Leu
P15	Trp-Arg-Arg-Trp-Phe-Tyr-Gln-Phe-Pro-Thr-Pro-Leu-Ala-Ala-Ala

In vitro P6.1 Breast Carcinoma Binding Assay. The ability of the P6.1 to recognize a native conformation of the erbB-2 expressed on a surface of human cancer cells was examined *in situ.* The expression of erbB-2 on MDA-MB-435 human breast carcinoma cells has been reported previously (4). In a double immunostaining experiments both Neu(9G6) anti-erbB-2 mouse monoclonal antibody and biotinylated P6.1 bound MDA-MB-435 human breast carcinoma cells (Figure 4 B and C). This is consistent with the data of ELISA experiments suggesting that antibody and peptide do not compete for the binding sites on the erbB-2 extracellular domain. The biotinylated control peptide did not bind MDA-MB-435 cells (data not shown). Neither Neu(9G6) anti-erbB-2 antibody nor biotinylated P6.1 bind T-24 human bladder carcinoma cells, which do not express T antigen on their surfaces (Figure 4 e and f).

P6.1 Binding Specificity and Affinity. The specificity of P6.1 peptide to the recombinant extracellular domain of erbB-2 was examined by slot-blotting and ELISA procedures. Affinity purified recombinant extracellular domain of erbB-2 and commercially available proteins: BSA, asialofetuin, and human IgG were immobilized on nitrocellulose paper in serial dilutions and visualized by the biotinylated peptide. At 15 – 7.5 ng immobilized protein, peptide P6.1 bound only erbB-2 and did not react with control proteins (Fig 5 A). The further analyses were performed to compare binding activity of P6.1 with the binding of control peptide. The binding affinity of the peptide to extracellular domain of erbB-2 was quantitatively evaluated using fluorescence titration experiments. Samples of protein were titered with increasing concentration of peptide. The quenching of tryptophane fluorescence was measured and used to generate binding curves (Fig 5 B). The data were collected at 2 μM, 4 μM and 6 μM concentrations of erbB-2. The equilibrium binding constant K_d = 30.21+-7.59 μM was determined using a single binding site curve-fitting procedure. The control peptide did not show any specific binding as assayed by fluorescence quench titration analysis (data not shown).

KEY RESEARCH ACCOMPLISHMENTS:

• The T antigen binding peptide P30 inhibits breast cancer tumor cell homotypic aggregation and heterotypic tumor cell endothelial cell adhesion *in vitro.*

• A six amino acid peptide, originally identified from a random peptide bacteriophage display library affinity selected with the extracellular domain of erbB-2, binds breast carcinoma cells that express the erbB-2 receptor on their surfaces.

REPORTABLE OUTCOMES:

- 2 manuscripts are in preparation.

-Funding for an additional postdoctoral fellow was obtained from the Cancer Research Center.

CONCLUSIONS:

Multicellular aggregate formation and adhesion of tumor cells to the endothelium are crucial events during early stages of cancer metastasis. Taken together our data indicated that β-galactoside and particularly T antigen mediated cell-cell interactions are important components of these events. To the best of our knowledge this is the first observation showing directly the accumulation of galectin-1 and galectin-3 at sites of contact between cancer and endothelial cells indicative of their active participation in adhesion of tumor cells to the endothelium. Strikingly different behavior of these two β-galactoside specific lectins reflects the complexity of the adhesion process. The accumulation of galectin-1 at the sites of cell-cell contacts predominantly on cancer cells and galectin-3 on endothelial cells suggests that several of their cognate ligands might be simultaneously involved here on both tumor and endothelial cells. Inhibition of tumor cell adhesion by the T antigen specific P-30 peptide, however, highlights an active role for this cell surface carbohydrate structure in cancer-endothelial cell interactions. The ability of a short synthetic peptide to effectively interfere with this line of intercellular communication may also be of functional significance for the development of new anti-adhesive therapies of cancer metastasis.

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APPENDIX I

FIGURES

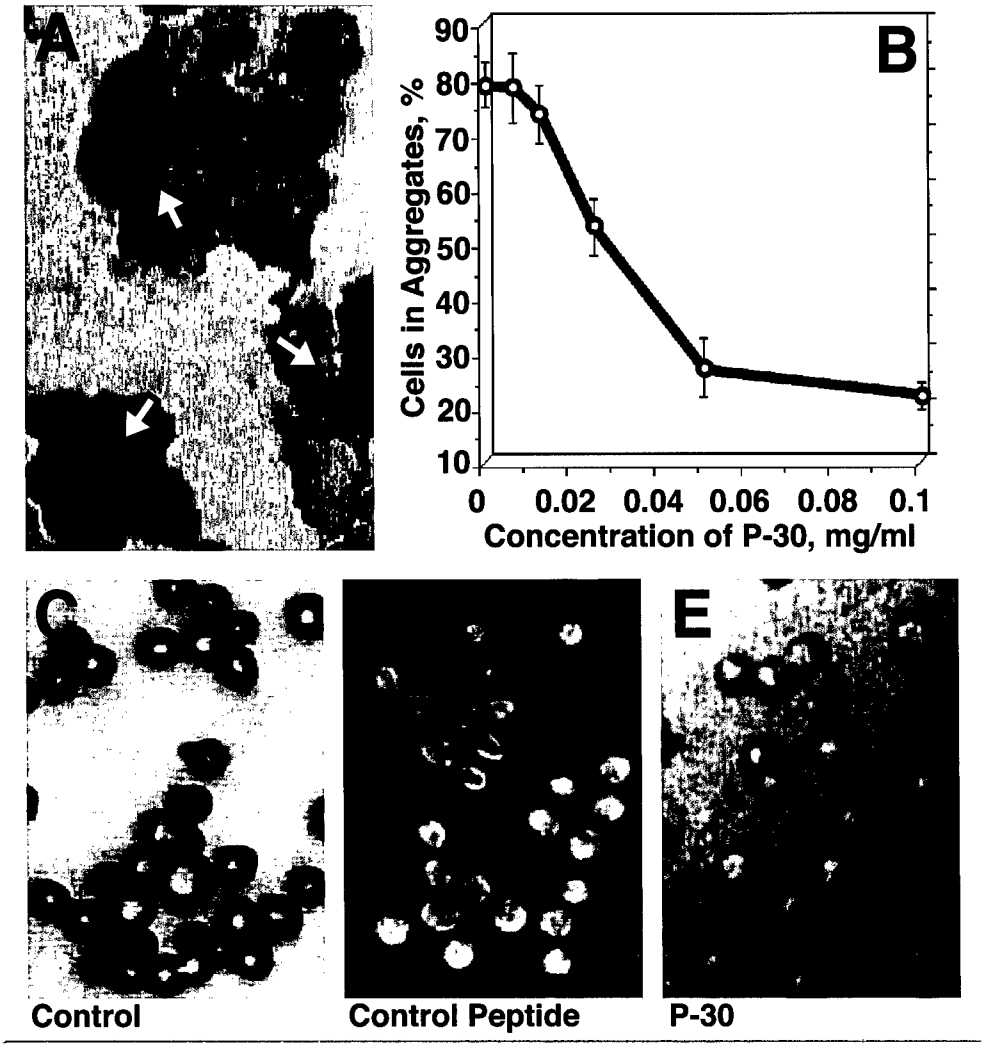


Figure 1

A. Direct binding of T antigen specific peanut (PNA) lectin to the MDA-MB-435 human breast carcinoma cells. Note the accumulation of T antigen at the sites of the cell-cell contact indicated by arrows. B. Dose dependent inhibition of spontaneous homotypic aggregation of the MDA-MB-435 human breast carcinoma cells by T antigen specific peptide P-30. C, D, and E - inhibition of spontaneous homotypic aggregation of MDA-MB-435 human breast carcinoma cells by 0.1 mg/ml of synthetic P-30 (E), but not by the same concentration of the control peptide (D) compared to the control (C).

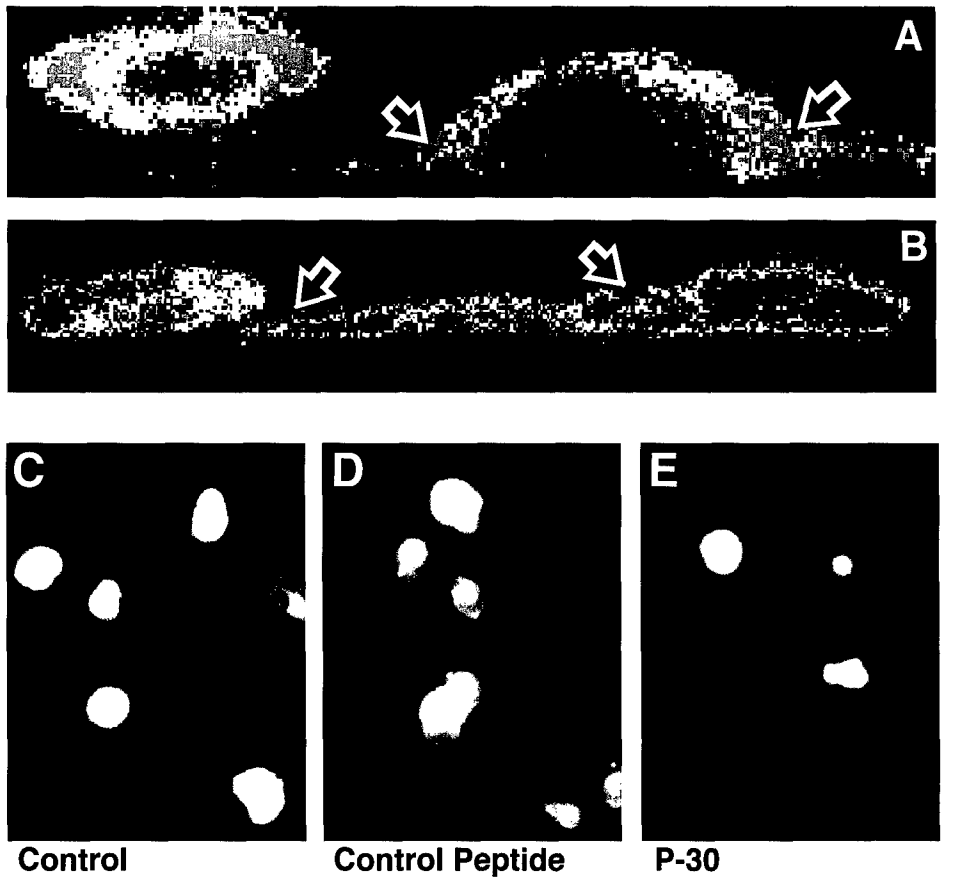


Figure 2

Involvement of the galectin-1 (A) and galectin-3 (B) in adhesion of the MDA-MB-435 human breast carcinoma cells to the monolayer of human umbilical endothelial cells as revealed by laser confocal microscopy. The X-Z sections shown were obtained as described in materials and methods using 60x lens. The images were thresholded and pseudocolored (galectin-1 – green, galectin-3 – red). Note the clusterization toward the cell contacts of galectin-1 on cancer cells (A) and galectin-3 on endothelial cells (B), indicated with arrows. The superimposed fluorescent photomicrographs of DiI labeled MDA-MB-435 breast cancer cells adhered to the monolayer of HUVEC cells (C, D, and E). Note inhibition of the adhesion by 0.1 mg/ml of the T antigen specific synthetic P-30 (E), but not by the same concentration of the control peptide (D) compared to the control (C).

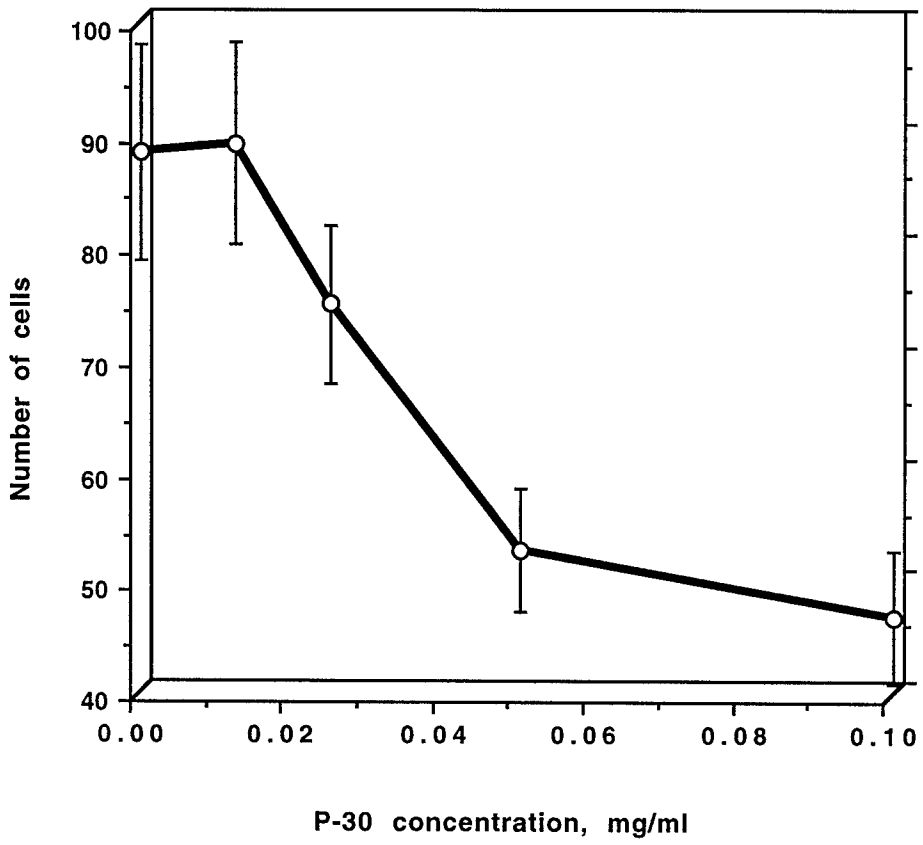


Figure 3

Dose dependent inhibition of the adhesion of MDA-MB-435 human breast carcinoma cells to the endothelium by synthetic T antigen specific peptide P-30. The maximum inhibitory effect (about 50%) was achieved at 0.1 mg/ml concentration of the peptide.

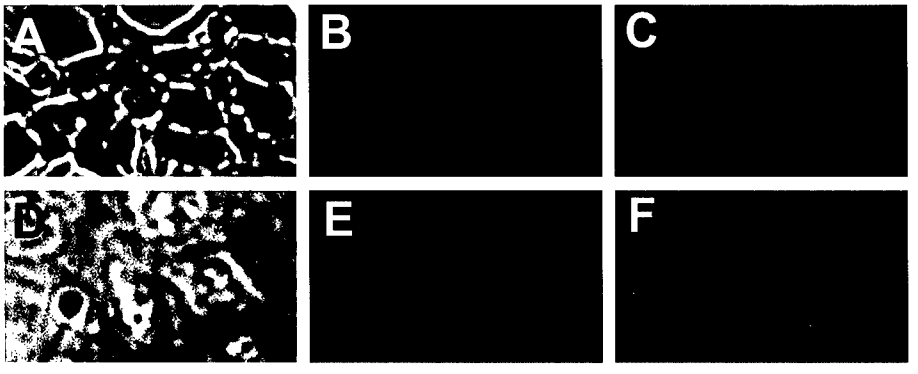
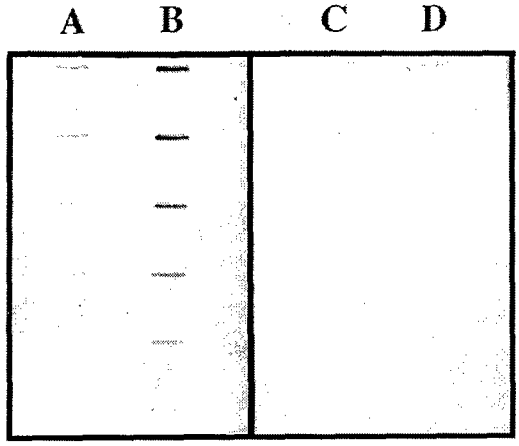


Figure 4

The ability of the P6.1 to recognize a native conformation of the *erbB-2* expressed on a surface of human cancer cells was examined in situ. In a double immunostaining experiments both Neu(9G6) anti-*erbB-2* mouse monoclonal antibody and biotinylated P6.1 bound human breast carcinoma cells MDA-MB-435 (Fig. 4 B and C respectively). This is consistent with the data of ELISA experiments suggesting that antibody and peptide do not compete for the binding sites on the erbB-2 extracellular domain. The biotinylated control peptide did not bind MDA-MB-435 cells (data not shown). Neither Neu(9G6) anti-*erbB-2* antibody no biotinylated P6.1 bind T-24 human bladder carcinoma cells, which do not express *erbB-2* (Fig. 4 E and F).



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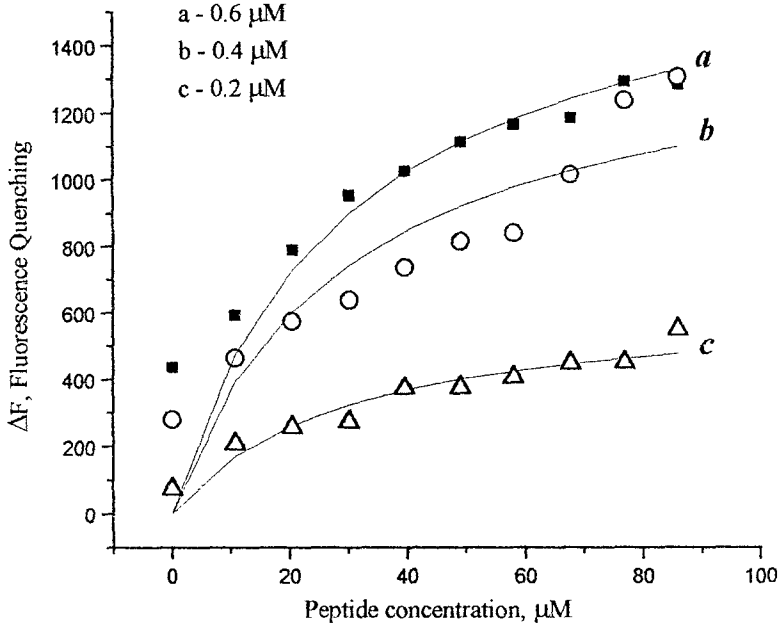

B

Fig5. **A – Slot-blot binding assay.** BSA (A), recombinant extracellular domain of erbB-2 (B), bovine asialofetuin (C) and human IgG (D) immobilyzed on nitrocellulose paper in different amounts were incubated with the biotinylated peptide and visualized by streptavidin-alkalyne phosphotase. **B – Determination of affinity constant by fluorescence titration.** Data used for generation of binding isoterms were obtained at three different concentration of erbB-2: 0.6 μM, 0.4 μM, 0.2 μM.

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